TNF RECEPTOR-SPECIFIC ANTIBODIES

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application is a division of U.S. application no. 08/476,862, filed June 7, 1995, which is a continuation-in-part of U.S. application no. 07/930,443, filed on August 19, 1992, and a continuation-in-part of U.S. application no. 08/321,685, filed October 12, 1994, now abandoned. The entire contents of both of said applications are hereby incorporated herein by reference. Application no. 07/930,443, filed on August 19, 1992, is a continuation of application no. 07/524,263, filed May 16, 1990, now abandoned.

FIELD OF THE INVENTION

[0002] The present invention relates to ligands to Tumor Necrosis Factor receptors (TNF-Rs) which inhibit the effect of TNF but not its binding to the TNF-Rs, as well as to ligands interacting with other receptors of the TNF/NGF family.

BACKGROUND OF THE INVENTION

[0003] Tumor necrosis factor (TNF) is a pleiotropic cytokine, produced by a number of cell types, mainly by activated macrophages. It is one of the principal mediators of the immune and inflammatory response. Interest in its function has greatly increased, recently, in view of evidence of the involvement of TNF in the pathogenesis of a wide range

of disease states, including endotoxin shock, cerebral malaria and graft-versus-host reaction. Since many of the effects of TNF are deleterious to the organism, it is of great interest to find ways of blocking its action on host cells. An evident

5   target for such intervention are the molecules to which TNF has to bind in order to exert its effects, namely the TNF-Rs. These molecules exist not only in cell-bound, but also in soluble forms, consisting of the cleaved extra-cellular domains of the intact receptors (see Nophar et al., EMBO

10   Journal, 9(10):3269-78, 1990). The soluble receptors maintain the ability to bind TNF, and thus have the ability to block its function by competition with surface receptors.

[0004] Another method of TNF inhibition based on the principle of competing with cell-bound molecules, is the use

15   of antibodies recognizing TNF receptors and blocking the ligand binding.

[0005] The cell surface TNF-Rs are expressed in almost all cells of the body. The various effects of TNF, the cytotoxic, growth-promoting and others, are all signaled by the TNF

20   receptors upon the binding of TNF to them. Two forms of these receptors, which differ in molecular size: 55 and 75 kilodaltons, have been described, and will be called herein p55 and p75 TNF-R, respectively. It should be noted, however,

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that there exist publications which refer to these receptors also as p60 and p80.

[0006] The TNF-Rs belong to a family of receptors which are involved in other critical biological processes. Examples of these receptors are the low affinity NGF receptor, which plays an important role in the regulation of growth and differentiation of nerve cells. Several other receptors are involved in the regulation of lymphocyte growth, such as CDw40 and some others. Another member of the family is the FAS receptor also called APO, a receptor which is involved in signaling for apoptosis and which, based on a study with mice deficient in its function, seems to play an important role in the etiology of a lupus-like disease. Herein, this family of receptors is called "TNF/NGF receptor family".

[0007] One of the most striking features of TNF compared to other cytokines, thought to contribute to the pathogenesis of several diseases, is its ability to elicit cell death. The cell-killing activity of TNF is thought to be induced by the p55 receptor. However, this p55 receptor activity can be assisted by the p75 receptor, through a yet unknown mechanism.

[0008] Parent application number 07/524,263 and European Patent publications 398,327 and 412,486 disclose antibodies to the soluble TNF-Rs. These antibodies were found to recognize the soluble TNF-Rs and to inhibit the binding of TNF to the

TNF-Rs on the cell surface. Monovalent F(ab) fragments blocked the effect of TNF, while intact antibodies were observed to mimic the cytotoxic effect of TNF. European patent publication 585,939 describes ligands interacting with a certain region in TNF-Rs.

SUMMARY OF THE INVENTION

[0009] The present invention provides a ligand to a member of the TNF/NGF receptor family, which binds either to the region of the fourth cysteine rich domain of such a receptor, or to the receptor between it and the cell membrane.

[0010] The region of the fourth cysteine rich domain will be called herein, for simplicity's sake, the "67 epitope" and the antibodies recognizing it the "group 67" antibodies. This region may extend between about amino acids pro-141 and thr-179 in the p75 TNF-R (residues 163-201 of SEQ ID NO:2) or a corresponding region in another member of the TNF/NGF family. More particularly, the region may extend between about amino acids pro-141 and cys-163 of the p75 TNF-R (residues 163-185 of SEQ ID NO:2) or a corresponding region in another member of the TNF/NGF family. The ligand downstream of the fourth cysteine rich domain includes the amino acid sequence between about thr-179 and about the end of the extracellular domain of the receptor (residues 201-257 of SEQ ID NO:2) or a corresponding region in another member of the TNF/NGF family.

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[0011] Preferably, the receptor is the TNF-R, in particular the p75 TNF-R.

[0012] One such ligand includes the amino acid sequence for the CDR region of the heavy chain of monoclonal antibody no. 67, and/or of the light chain thereof.

[0013] Another such ligand includes the amino acid sequence for the CDR region of the heavy chain of monoclonal antibody no. 81, and/or the light chain thereof.

[0014] Yet another such ligand includes the amino acid sequence or antibody against the "stalk" region, i.e., from about amino acid thr-181 to about amino acid 235-asp.

[0015] The ligands may comprise, for example, proteins, peptides, immunoadhesins, antibodies or other organic compounds.

[0016] The proteins may comprise, for example, a fusion protein of the ligand with another protein, optionally linked by a peptide linker. Such a fusion protein can increase the retention time of the ligand in the body, and thus may even allow the ligand-protein complex to be employed as a latent agent or as a vaccine.

[0017] The term "proteins" includes muteins and fused proteins, their salts, functional derivatives and active fractions

[0018] "Functional derivatives" as used herein cover derivatives of the ligands and their fused proteins and muteins, which may be prepared from the functional groups which occur as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e., they cannot destroy the activity of the ligand and do not confer toxic properties on compositions containing it. These derivatives may, for example, include polyethylene glycol side-chains which .may mask antigenic sites and extend the residence of the ligands in body fluids. Other derivatives include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives of free amino groups of the amino acid residues formed with acyl moieties (e.g., alkanoyl or carbocyclic aroyl groups) or O-acyl derivatives of free hydroxyl groups (for example, that of seryl or threonyl residues) formed with acyl moieties.

[0019] As "active fractions" of the ligands, its fused proteins and its muteins, the present invention covers any fragment or precursors of the polypeptide chain of the ligand alone or together with associated molecules or residues linked thereto, e.g., sugar or phosphate residues, or aggregates of the protein molecule or the sugar residues by themselves,

provided said fraction has the same biological and/or

pharmaceutical activity.

[0020] As used herein the term "muteins" refers to analogs

of the proteins, peptides and the like in which one or more or

5   the amino acid residues of the protein found to bind are

replaced by different amino acid residues or are deleted, or

one or more amino acid residues are added to the original

sequence, without changing considerably the activity of the

resulting product. These muteins are prepared by known

10   synthesis and/or by size-directed mutagenesis techniques, or

any other known technique suitable therefor.

[0021] The term "fused protein" refers to a polypeptide

comprising the ligands or a mutein thereof fused with another

protein which has an extended residence time in body fluids.

15   The ligands may thus be fused to another protein, polypeptide

or the like, e.g., an immunoglobulin or a fragment thereof.

[0022] The term "salts" herein refers to both salts or

carboxyl groups and to- acid addition salts of amino groups of

the ligands, muteins and fused proteins thereof. Salts of a

20   carboxyl group may be formed by means known in the art and

include inorganic salts, for example, sodium, calcium,

ammonium, ferric or zinc salts, and the like, and salts with

organic bases as those formed, for example, with amines, such

as triethanolamine, arginine or lysine, piperidine, procaine

and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid.

[0023] The peptides include peptide bond replacements and/or peptide mimetics, i.e., pseudopeptides, as known in the art (see, e.g., Proceedings of the 20th European Peptide Symposium, ed. G. Jung, E. Bayer, pp. 289-336, and references therein), as well as salts and pharmaceutical preparations and/or formulations which render the bioactive peptide(s) particularly suitable for oral, topical, nasal spray, ocular, pulmonary, I.V. or subcutaneous delivery, depending on the particular treatment indicated. Such salts, formulations, amino acid replacements and pseudopeptide structures may be necessary and desirable to enhance the stability, formulation, deliverability (e.g., slow release, prodrugs), or to improve the economy of production, as long as they do not adversely affect the biological activity of the peptide.

[0024] Besides substitutions, three particular forms of peptide mimetic and/or analogue structures of particular relevance when designating bioactive peptides, which have to bind to a receptor while risking the degradation by proteinases and peptidases in the blood, tissues and elsewhere, may be mentioned specifically, illustrated by the

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following examples: Firstly, the inversion of backbone chiral

centres leading to D-amino acid residue structures may,

particularly at the N-terminus, lead to enhanced stability for

proteolytical degradation without adversely affecting

5   activity. An example is given in the paper "Tritriated D-ala[1]-

Peptide T Binding", Smith C.S. et al., Drug Development Res.

15, pp. 371-379 (1988). Secondly, cyclic structure for

stability, such as N to C interchain imides and lactams (Ede

et al. in Smith and Rivier (Eds.) "Peptides: Chemistry and

10   Biology., Escom, Leiden (1991), pp. 268-270), and sometimes

also receptor binding may be enhanced by forming cyclic

analogues. An example of this is given in "Confirmationally

restricted thymopentin-like compounds", US Pat. 4,457,489

(1985), Goldstein, G. et al. Thirdly, the introduction of

15   ketomethylene, methylsufide or retroinverse bonds to replace

peptide bonds, i.e., the interchange of the CO and NH moieties

are likely to enhance both stability and potency. An example

of this type is given in the paper "Biologically active

retroinverso analogues of thymopentin", Sisto A. et al in

20   Rivier, J.E. and Marshall, G.R. (eds) Peptides, Chemistry,

Structure and Biology", Escom, Leiden (1990), pp. 722-773).

[0025] The peptides of the invention can be synthesized by

various methods which are known in principle, namely by

chemical coupling methods (cf. Wunsch, E: "Methoden der

organischen Chemiet", Volume 15, Band 1 + 2, Synthese von

Peptiden, thime Verlag, Stutt (1974), and Barrany, G.;

Marrifield, R.B.: "The Peptides", eds. E. Gross, J.

Meienhofer, Volume 2, Chapter 1, pp. 1-284, Academic Press

5   (1980)), or by enzymatic coupling methods (*cf.* Widmer, F.

Johansen, J.T., Carlsberg Res. Commun., Vol.44, pp. 37-46

(1979), and Kullmann, W.: "Enzymatic Peptide Synthesis", CRC

Press Inc. Boca Raton, Fl. (1987), and Widmer, F., Johansen,

J.T. in "Synthetic Peptides in Biology and Medicines", eds.

10 Alitalo, K., Partanen, P., Vatieri, A., pp.79-86, Elsevier,

Amsterdam (1985)), or by a combination of chemical and

enzymatic methods if this is advantageous for the process

design and economy.

[0026] A cysteine residue may be added at both the amino

15 and carboxy terminals of the peptide, which will allow the

cyclization of the peptide by the formation of a disulphide

bond.

[0027] Any modifications to the peptides of the present

invention which do not result in a decrease in biological

20 activity are within the scope of the present invention.

[0028] There are numerous examples which illustrate the

ability of anti-idiotypic antibodies (anti-Id Abs) to an

antigen to function like that antigen in its interaction with

animal cells and components of cells. Thus, anti-Id Abs to a

peptide hormone antigen can have hormone-like activity and interact specifically with a mediator in the same way as the receptor does. (For a review of these properties see: Gaulton, G.N. and Greane, M.I. 1986. Idiotypic mimicry of biological receptors, Ann. Rev. Immunol. Vol. 4, pp. 253-280; Sege K. and Peterson, P.A., 1978, Use of anti-idiotypic antibodies as cell surface receptor probes, Proc. Natl. Acad. Sci. U.S.A., Vol. 75, pp. 2443-2447).

[0029] It is expected from this functional similarity of anti-Id Ab and antigen, that anti-Id Abs bearing the internal image of an antigen can induce immunity to such an antigen. (See review in Hiernaux, J.R., 1988, Idiotypic vaccines and infectious diseases, Infect. Immun., Vol. 56, pp. 1407-1413).

[0030] It is, therefore, possible to produce anti-idiotypic antibodies to the peptides of the present invention which will have similar biological activity.

[0031] Accordingly, the present invention also provides anti-idiotypic antibodies to the peptides of the present invention, the anti-idiotypic antibody being capable of inhibiting TNF toxicity, but not its binding to the receptor.

[0032] The individual specificity of antibodies resides in the structures of the peptide loops making up the Complementary Determining Regions (CDRs) of the variable domains of the antibodies. Since in general the amino acid

sequence or the CDR peptides of an anti-Id Ab are not

identical to or even similar to the amino acid sequence of the

peptide antigen from which it was originally derived, it

follows that peptides whose amino acid sequence in quite

5   dissimilar, in certain contexts, can take up a very similar

three-dimensional structure. The concept of this type of

peptide, termed a "functionally equivalent sequence" or

mimotope by Geyson is known. (Geyson, H.M. et al, 1987,

Strategies for epitope analysis using peptide synthesis., J.

10   Immun. Methods, Vol. 102, pp. 259-274).

[0033] Moreover, the three-dimensional structure and

function of the biologically active peptides can be simulated

by other compounds, some not even peptidic in nature, but

which nevertheless mimic the activity of such peptides. This

15   field is summarized in a review by Goodman, M. (1990),

(Synthesis, Spectroscopy and computer simulations in peptide

research, Proc. 11th American Peptide Symposium published in

Peptides-Chemistry Structure and Biology, pp. 3-29; Eds.

Rivier, J.E. and Marshall, G.R. Publisher Escom).

20   [0034] It is also possible to produce peptide and non-

peptide compounds having the same three-dimensional structure

as the peptides of the present invention. These "functionally

equivalent structures" or "peptide mimics" will react with

antibodies raised against the peptide of the present invention and may also be capable of inhibiting TNF toxicity.

[0035] Accordingly, a further embodiment of the present invention provides a compound the three-dimensional structure of which is similar as a pharmacophore to the three-dimensional structure of the peptides of the present invention, the compound being characterized in that it reacts with antibodies raised against the peptides of the present invention and that the compound is capable of inhibiting TNF toxicity.

[0036] More detail regarding pharmacophores can be found in Bolin et al., p. 150, Polinsky et al., p. 287, and Smith et al., p. 485, in Smith and Rivier (eds.) "Peptides: Chemistry and Biology", Escom, Leiden (1991).

[0037] All of the molecules (proteins, peptides, etc.) may be produced either by conventional chemical methods, as described herein, or by recombinant DNA methods.

[0038] All of the molecules (proteins, peptides, etc.) may be produced either by conventional chemical methods, as described herein, or by recombinant DNA methods.

[0039] The invention also provides DNA molecules encoding the ligands according to the invention, vectors containing them and host cells comprising the vectors and capable of expressing the ligands according to the invention.

[0040] The host cell may be either prokaryotic or eukaryotic.

[0041] The invention further provides DNA molecules hybridizing to the above DNA molecules and encoding ligands having the same activity.

[0042] The invention also provides pharmaceutical compositions comprising the above ligands which are useful for treating diseases induced or caused by the effects of TNF, either endogenously produced or exogenously administered.

[0043] The invention also provides for using the ligands according to the invention for increasing the inhibitory effect of a soluble receptor of the TNF/NGF receptor family. As stated above, the soluble receptors, especially those of TNF, have the ability to block the function of TNF by binding it in competition with the surface receptors. Application of a ligand according to the invention together with a soluble receptor is, therefore, expected to increase the inhibitory effect of the soluble receptor.

BRIEF DESCRIPTION OF THE FIGURES

[0044] Figure 1 shows the results of the test by which epitope 67 was mapped.

[0045] Figure 2 shows the nucleotide (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequences of the p75 receptor, TBP-II and transmembranal domains are boxed and

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shaded.   The region recognized by the group 67 antibodies is

underlined, and the region recognized by the anti-stalk

antibodies is underlined by a broken line.

[0046] Figure 3 shows the inhibitory effect of the 67 and

5   anti-stalk antibodies on TNF function in HeLa cells.

[0047] Figure 4 shows that antibodies against the upper

part of extracellular domain of the p75 TNF-R are signaling in

the HeLa cells.

[0048] Figure 5 shows that antibodies against the upper

10   part of the extracellular domain of the p75 TNF-R do not

signal in A9 cells which express the human p75 TNF-R.

Antibodies of the 67 group do have, though, an inhibitory

effect on TNF function in them (Fig. 6).

[0049] Figure 6 shows that antibodies against the upper

15   part of the extracellular domain of the p75 TNF-R inhibit TNF

function in A9 cells.

[0050] Figure 7 shows that antibodies against the upper

part of the extracellular domain of the p75 TNF-R do not

signal in A9 cells which express the cytoplasmically truncated

20   p75 TNF-R. Antibodies of the 67 group do have, though, an

inhibitory effect on TNF function in them (not shown).

[0051] Figure 8 shows that antibodies against the 67

epitope impede TNF dissociation from p75 TNF-R.

[0052] <u>Figure 9</u> shows the sequence homology between several members of the TNF/NGF receptor family (residues 3-155 of hu p55 TNF-R (SEQ ID NO:3); residues 39-201 of hu p75 TNF-R (SEQ ID NO:4); residues 31-149 of hu FAS (SEQ ID NO:5); residues 3-

5   161 of hu NGF-R (SEQ ID NO:6); residues 25-187 of hu CDw40 (SEQ ID NO:7); and residues 25-164 of rat Ox40 (SEQ ID NO:8)).

DETAILED DESCRIPTION OF THE INVENTION

[0053] TNF, as stated above, is a cytokine which initiates its effect on cell function by binding to two specific cell

10   surface receptors: the p55 and p75 receptors.   Binding of antibodies to the extracellular domain of these receptors can interfere with its effect.   However, as shown in a number of studies, antibodies binding to the extracellular domain of the receptors can also trigger the effects of TNF by inducing

15   aggregation of the p55 receptors, as well as by inducing aggregation of the p75 receptors. (Engelmann, et al. J. Biol. Chem., Vol. 265, No. 24, pp. 14497-14504, 1990; and unpublished data).

[0054] As disclosed in patent application no. 103051,

20   antibodies binding to one particular region in the p75 receptor are not mimetic but rather inhibitory to the signaling for the cytocidal effect by this receptor.   This, in spite of the fact that when binding to this region, these antibodies do not block TNF binding, but rather increase it to

some extent. In application no. 106271 this region is more

particularly identified as extending between cys-163 and thr-

179, in the fourth cysteine rich domain of the receptor. The

present invention reveals that the region recognized by

5 certain other antibodies is the region extending downstream of

thr-181 and upstream of cys-163 to about cys-142 in the

extracellular domain-of the p75 receptor.

[0055] The present invention also reveals that the so-

called "stalk-antibody" recognizes a region downstream of the

10 fourth cysteine rich domain, more particularly the region

extending from about amino acid 181 to about amino acid 235.

[0056] It was also found in accordance with the present

invention that, in case of the "67 epitope" antibodies, the

divalent antibodies have an effect which mimics TNF action,

15 while the monovalent fragments, such as F(ab), inhibit the

cytotoxic effect of TNF.

[0057] Based on these findings, small molecular weight

compounds, such as peptides or mimetic compounds, which will

either inhibit the function of the p75 receptor, or enhance

20 it, can be defined.

[0058] In view of these findings, as well as the close

similarity of the receptors in this particular family, this

invention relates also to ligands which bind to the same

regions in the extracellular domain of the various other

members of the TNF/NGF receptor family and modulate the function of the other receptors, similarly to the modulation of the function of TNF. In this receptor family, the localization of cysteines in the extracellular domain and the spacing is highly conserved. Certain members of this family, e.g., CDw40, exhibit particularly high similarity to the p75 receptor. Particularly in such receptors, ligands binding to these regions are expected to have effects similar to the effect of the ligands according to the present invention on the p75 receptor.

[0059] Recombinant production of the ligands is carried out by known methods commonly employed in the art.

[0060] The invention is illustrated by the following non-limiting examples:

EXAMPLE 1: Monoclonal Antibodies to TBP-II

Production of the Monoclonal Antibodies

[0061] Female Balb/C mice (8 weeks old) were injected with 1 µg purified TBP-II in an emulsion of complete Freund's adjuvant into the hind foot pads, and three weeks later, subcutaneously into the back in incomplete Freund's adjuvant. The other injections were given in weekly intervals, subcutaneously in PBS. Final boosts were given 4 days (i.p.) and 3 days (i.v.) before the fusion with 9.0 µg of TBP-I in PBS. Fusion was performed using NSO/Mr cells and lymphocytes

prepared from both the spleen and the local lymphocytes of the

hind legs as fusion partners. The hybridomas were selected in

DMEM. supplemented with HAT, 15% horse serum and gentamycin 2

μg/ml. Hybridomas that were found to produce antibodies to

5   TBP-1 were subcloned by the limiting dilution method and

injected into Balb/C mice that had been primed with pristane

for the production of ascites. Immunoglobulins were isolated

from the ascites by ammonium sulfate precipitation (50%

saturation) and then dialyzed against PBS containing 0.02%

10   azide. Purity was approximately 60% as estimated by analysis

on SDS-PAGE and staining with Coomassie blue. The isotypes of

the antibodies were defined with the use of a commercially

available ELISA kit (Amersham, U.K.).

[0062] Several positive clones were obtained, subcloned for

15   further studies and characterized. Some of the isolated

subclones with their isotype and binding of TBP-II in inverted

RIA are listed in Table I.

TABLE I
Subclones Producing Monoclonal Antibodies to TBP-II

Clone Number	Screening with iRIA [CPM]	Screening of subclone with iRIA [CPM]	Isotype
13.11	31800	31000	IgG_1
.12		31500	IgG_1
.13		31100	IgG_1
14.1	15300	15400	IgG_{2a}
.6		16200	IgG_{2a}
.7		15300	IgG_{2a}
20.2	12800	14200	IgG_{2b}
.5		14300	IgG_{2b}
.6		14800	IgG_{2b}
22.7	20400	20000	IgG_1
.8		19300	IgG_1
27.1	1800	27000	IgG_{2a}
.3		25000	IgG_{2a}
.9		28000	IgG_{2a}
32.4	11315	10900	IgG_{2b}
.5		10700	IgG_{2b}
.6		11200	IgG_{2b}
33.1	18400	11400	IgG_1
.3		10500	IgG_1
.4		14800	IgG_1
36.1	27500	26600	IgG_{2a}
.5		24900	IgG_{2a}
.6		24900	IgG_{2a}
41.3	13800	18100	IgG_1
.7		18100	IgG_1
.10		18800	IgG_1
67.1	16800	10900	IgG_{2a}
.16		10800	IgG_{2a}
.17		10900	IgG_{2a}
70.2	15100	5100	IgG_{2a}
.3		5200	IgG_{2a}
.4		5300	IgG_{2a}
77.2	15300	11800	IgG_{2b}
78.9	25300	21400	IgG_{2a}
82.1	17600	25900	IgG_1
.4		25700	IgG_1
.10		26400	IgG_1
86.2	8800	12200	IgG_{2b}
.5		12600	IgG_{2b}
.11		12800	IgG_{2b}
19.6		29700	IgG_{2a}
.9		28900	IgG_{2a}

[0063] Hybridomas TBP-II 13-12 and TBP-II 70-2 were deposited with the Collection Nationale de Cultures de Microorganismes (CNCM), Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris CEDEX 15, France on March 12, 1990, and were assigned No. I-929 and No. I-928. respectively.

EXAMPLE 2: Inverted Radioimmunoassay (iRIA) for the Detection of the Monoclonal Antibodies to TBP-II

[0064] This assay was used for estimating the level of the anti-TBP antibodies in the sera of the immunized mice and for screening for the production of the antibodies by hybridomas. PVC, 96-well microtiter plates (Dynatech 1-220-25) were coated for 12 hr at 4°C with affinity purified goat anti mouse F(ab) immunoglobulins (Biomakor, Israel 10 µg/ml in PBS containing 0.02: NaN_3), then blocked for 2 hr at 37°C with 0.52 BSA in PBS supplemented with 0.05% Tween 20 (Sigma) and 0.02% NaN_3 (blocking buffer) and washed 3 times with PBS containing 0.05% Tween 20 and 0.02% NaN_3 (washing buffer). Serum samples, in serial dilutions, or samples of hybridoma growth media (50 µl) were applied into the wells for 2 hr at 37°C. The plates were rinsed with washing buffer and ^{125}I-labelled TBP-I (10,000 cpm, in blocking buffer) was applied into the wells. After further incubation of 2 hr at 37°C, the plates were washed and the amount of label which bound to individual wells was determined in the gamma-counter.

EXAMPLE 3:   The Use of Anti-TBP-II Antibodies for Affinity Chromatography

[0065] Antibodies against TBP-II can be utilized for the purification of TBP-II by affinity chromatography, according to the following procedure. The monoclonal antibodies for affinity chromatography were selected by testing their binding capacity for the radiolabeled antigen in a solid phase radio immunoassay. Ascites from all hybridomas was purified by ammonium sulfate precipitation at 50% saturation followed by extensive dialysis against PBS. PVC 96-well plates were coated with the purified McAbs, and after blocking the plates with PBS containing 0.5% BSA, 0.05% Tween 20 (Sigma) and 0.02% NaN$_3$, the wells were incubated with 50,000 cpm ^{125}I-TNF for 2 hr at 37°C, then washed and the radioactivity which had bound to each well was quantitated in the gamma-counter. The antibodies with the highest binding capacity were examined for their performance in immunoaffinity chromatography.

[0066] Polyacryl hydrazide agarose was used as resin to immobilize the antibodies. The semipurified immunoglobulins were concentrated and coupled to the resin as specified by Wilchek and Miron, Methods in Enzymology 34:72-76, 1979. Three monoclonal antibodies against TBP-I, clones 16, 20, and 34 were tested in these experiments. Antibody columns of 1 ml bed were constructed. Before use, all columns were subjected to 10 washes with the elusion buffer, each wash followed by

neutralization with PBS. Then the columns were loaded with 120 ml of concentrated urinary proteins in PBS with 0.02% NaN_3. The flow rate of the columns was adjusted to 0.2 to 0.3 ml per minute. After loading, the columns were washed with 50 ml PBS

5   and then eluted with a solution containing 50 mM citric acid, pH 2.5, 100 mM NaCl and 0.02% NaN_3. Fractions of 1 ml were collected. Samples of the applied urinary proteins, the last portion of the wash (1 ml) and of each elusion fraction (8 fractions of 1 ml per column) were taken and tested for

10   protein concentration and activity in the bioassay for T3P-II. According to the protein measurements before and after coupling of the antibodies to hydrazide agarose, the amounts of immunoglobulin bound to the columns ranged from 7 to 10 mg/ml agarose. All protein measurements were done according

15   to a micro-flurescamin method in comparison to a standard solution containing 100 µg BSA/ml (Stein, S. and Moschera. J., Methods Enzymol. 79:7-16, 1981).

EXAMPLE 4:   Determination of TBP-II Using Anti-TBP-II Antibodies

20   [0067] The levels of TBP-II in the sera of healthy individuals, patients with cancer or systemic lupus erythematosus (SLE) and of pregnant women at term were determined by an ELISA method employing a monoclonal antibody to TBP-IO coating the plates. 50 µl of each sample was added

and after a 2.5 hr incubation at 37°C the wells were washed

with a solution of PBS, Tween 0.05% and sodium azide 0.02%,

after which a rabbit anti-TBP-II polyclonal antibody was added

for 2.5 hr at 37°C. Then the wells were washed again (no

5   azide) and goat anti-rabbit horseradish peroxidase-coupled

antibody was added for 2 hr. Following this incubation, and

washing, an ABTS buffer was added and optical density (O.D.)

read 30 min. later at 600 nm.

[0068] The normal levels of TBP-II in human serum of

10   healthy individuals as determined by the ELISA method are

1.48±0.46 ng/ml.

EXAMPLE 5:   Epitope Mapping of TBP-II by Cross Competition
             Analysis with Monoclonal Antibodies (mAbs) to TBP-
             II

15   [0069] PVC 96-well microtiter plates were coated as

described above, with purified mAbs to TBP-II (25 µg/ml).

Following rinsing and blocking, samples of ^{125}I-labelled T3P-II

(100,000 cpm per well) which had been preincubated for 2 hr,

at 37°C with the same or a different monoclonal antibody to

20   TBP-II (at 1 µg/ml) were put into the wells; the plates were

incubated overnight at 4°C, washed and the radioactivity bound

to each well was determined by gamma counting. The results

are expressed as percent of the control values (TBP-II binding

in the absence of competing mAbs).

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[0070] The results are depicted in Table II. The

monoclonal antibodies are indicated by the clone numbers in

the first row and ~n left column. Low percent binding values

indicate that the two antibodies compete for each other's

5   epitope on TBP-II, while higher values indicate that they bind

to different epitopes. Non-competitive antibodies are

suitable for use in double-sandwich ELISA, e.g., clones 13 and

70.

TABLE II

Cross Competition Analysis with Monoclonal Antibodies to TBP II

Competitor Antibody	Solid Phase Antibodies															
	13	14	19	20	22	27	32	33	36	41	67	70	77	78	82	86
13	4	64	53	73	31	51	161	35	177	72	131	128	77	102	50	101
14	119	20	90	13	13	84	156	11	132	173	134	113	14	70	89	179
19	103	28	7	19	11	5	144	11	144	133	179	123	18	5	85	126
20	119	17	93	14	10	88	149	9	135	170	137	135	16	70	101	181
22	109	26	94	22	13	82	128	12	115	164	136	114	17	68	98	167
27	106	23	11	27	14	8	145	17	152	133	196	136	24	8	82	125
32	150	267	150	291	156	186	14	163	139	200	205	18	294	143	103	226
33	115	19	98	23	16	86	133	12	118	156	120	114	24	78	90	155
36	155	262	168	271	144	185	167	158	12	169	223	135	265	158	93	150
41	117	119	119	118	101	109	118	76	93	9	179	107	106	111	8	9
67	112	138	125	141	125	157	136	107	138	213	30	117	120	127	106	236
70	150	246	150	255	145	166	4	162	166	217	204	6	232	132	107	234
77	121	18	98	15	13	78	148	11	145	184	142	132	18	66	103	184
78	118	20	9	26	10	6	153	13	157	137	183	131	19	6	94	172
82	107	110	130	116	112	121	128	89	90	8	162	102	121	113	8	7
86	122	181	125	166	126	129	131	120	86	18	253	109	152	125	20	17
100% value	31582	3958	2057	5437	2947	17395	25923	3525	6368	8042	4368	24113	5887	22222	11608	9703

EXAMPLE 6: Mapping of the Epitope 67 of the p75 TNF-R

[0071] In order to compare the function of the 67 group
antibodies, not only to antibodies which bind to the receptor
at the 67 epitope region, but also to antibodies that bind to
the receptor downstream to that epitope region, we immunized
rabbits with a chimeric construct corresponding to the region
extending downstream to the 32 epitope (amino acids 181 to
235; the "stalk" region), linked to MBP. The rabbits
developed antibodies which bound to the chimera with which
they were immunized as well as to the intact p55 TNF receptor.
These antibodies were affinity purified by binding to the
chimeric protein, linked to an Affigel 10 column, and tested
for effect on TNF function and binding. (The affinity
purified antibody preparation was termed "318").
The mapping of epitope 67 was carried out by examining the
ability of antibodies number 67 and 13 (an antibody that binds
to the upper part of the extracellular domain of the p75 TNF-
R) as well as antiserum 318, to immunoprecipitate the
following methionine-labeled soluble p75 TNF-R mutants: WT- a
receptor extending from amino acid 22 to amino acid 234, D4D-
a receptor like WT, from which the 4th cysteine-rich domain
has been deleted (amino acids 141 to 180). The receptors were
produced by *in vitro* transcription of cDNAs encoding them
(from the Bluescript vector, using the T7 promoter) followed

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by *in vitro* translation using the Promega TnT kit. The

immunoprecipitated proteins were analyzed by SDS PAGE,

followed by autoradiography. (A) Immunoprecipitation of WT.

All antibodies were effective. (B) Immunoprecipitation of

5   D4D. Only antibodies 13 and 318 were effective. The findings

indicate that epitope 67 is located at the upper part of the

4th cysteine rich domain, within about amino acids 141 to 180.

**EXAMPLE 7:   Titration of the Inhibitory Effect of the Group 67
Antibodies and the Anti-Stalk Antibodies on TNF**
10   **Function**

[0072] As shown in Figure 3, the protective effect of the

different antibodies studied on the cytocidal effect of TNF on

HeLa p75.3 cells was found to vary depending on the particular

antibody used: antibodies 32 and antiserum 313 and their Fab

15   monovalent fragments, which protect, antibody 67, which

protects as Fab monovalent fragment and enhances TNF

cytotoxicity in its divalent form, and antibody 13 (which

binds to the upper part of the extracellular domain of the

-75-R) which enhances the cytocidal effect of TNF (p75.3 cells

20   are HeLa cells transfected with the full length p75 TNF-R).

**EXAMPLE 8:   The Inhibitory Effect of the Group 67 and Anti-
Stalk Antibodies Is Independent of the Expression
and Function of the Intracellular Domain of the
p75 TNF-R**

25   [0073] In HeLa cells which over-express the p75 TNF-R,

antibodies against the upper part of the extracellular domain

of the receptor have a cytocidal effect, synergistic with that
or antibodies against the p55-R (Fig. 4). However, these
antibodies do not have such an effect in A9 cells which
express either tine full-length or cytoplasmically-truncated
human p75 TNF-R (Figs. 5 and 7, respectively). However,
antibodies which bind to the lower part of the receptor did
show inhibitory effect on TNF function even in these cells,
irrespective of whether the cells expressed the full-length or
the cytoplasmically truncated receptor (see Fig. 6 as well as
data not shown).

**EXAMPLE 9:   Effect of the various antibodies on the
                dissociation of TNF form p75 TNF**

[0074] Figure 8 shows a comparison of the rate of the
dissociation of TNF from the p55 TNF-R, as assessed by
measuring the dissociation of radiolabeled TNF from mouse A9
cells expressing transfected human p55 TNF-R (A9D2 cells, in
which over 90% of the cell-bound TNF is associated with the
human p55 TNF-R) and from the HeLa p75.3 cells, in which most
of the bound TNF is associated with the over-expressed p75
TNF-R. As opposed to the very slow dissociation of TNF from
the p55 TNF-R, TNF dissociates rather rapidly from the p75
TNF-R.

EXAMPLE 10

[0075] Figure 9 shows the internal cysteine rich repeats in the extracellular domains of the two TNF-Rs and their alignment with the homologous repeats in the extracellular domain of the human FAS, nerve growth factor receptor (NGF) and CDw40, as well as rat Ox40. The amino acid sequences (one letter symbols) are aligned for maximal homology. The positions of the amino acids within the receptors are denoted in the left hand margin.

Deposit Information

[0076] Hybridomas TBP-II 67 and 81 were deposited with the Collection National de Cultures de Microorganismes (CNCM), Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France, on October 11, 1993 and assigned Nos. I-1368 and I-1369, respectively.